Molecular analysis of a new cytoplasmic male sterile genotype in sunflower
Spassova, Mariana; Christov, Michail; Bohorova, Natasha; Petrov, Peter; Dudov, Kalin; Atanassov, Atanas; Nijkamp, H. John J.; Hille, Jacob

Published in:
FEBS Letters

DOI:
10.1016/0014-5793(92)80350-P

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
1992

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.
Molecular analysis of a new cytoplasmic male sterile genotype in sunflower

Mariana Spassova, Michail Christov, Natasha Bohorova, Peter Petrov, Kalin Dudov, Atanas Atanassov, H. John J. Nijkamp and Jaques Hillem

"Institute of Genetic Engineering, 2232 Kostinbrod-2, Bulgaria, "Institute of Wheat and Sunflower 'Dobruda', General Toshevo, Bulgaria, "Institute of Genetics, 1113 Sofia, Bulgaria and Free University, Department of Genetics, De Boelelaan 1087, NL-1081 HV Amsterdam, The Netherlands

Received 3 September 1991; revised version received 6 December 1991

Mitochondrial DNA from 1 fertile and 6 cytoplasmic male sterile (CMS) sunflower genotypes was studied. The CMS genotypes had been obtained either by specific crosses between different Helianthus species or by mutagenesis. CMS-associated restriction fragment length polymorphisms (RFLPs) were found in the vicinity of the atpA locus, generated by various restriction enzymes. The organization of the mitochondrial genes 26S rRNA, 18S+5S rRNA and coxII was investigated by Southern blot analysis. These genes have similar structures in fertile and all studied sterile sources. Using the atpA probe, 5 from the 6 investigated CMS genotypes showed identical hybridization patterns to the Petunia CMS line, which is used in all commercial sunflower hybrids. Only 1 cytoplasm derived from an open pollination of Helianthus annuus ssp. texanus, known as ANT, contained a unique mitochondrial DNA fragment, which is distinguishable from the fertile and sterile Petunia CMS genotypes and from all investigated CMS genotypes. Male fertility restoration and male sterility maintenance of the ANT line are different from the Petunia CMS system, which is a confirmation that a novel CMS genotype in sunflower has been identified.

Sunflower; Mitochondrial genome; Cytoplasmic male sterility; RFLP; atpA locus

1. INTRODUCTION

Cytoplasmic male sterility (CMS) is a maternally inherited trait in higher plants that results in the inability of the mature plant to produce functional pollen, but it does not affect female fertility [1]. In sunflower a CMS genotype was obtained from an interspecific cross between Helianthus petiolaris and H. annuus which was first described by Leclercq [2]. The subsequent identification of male fertile lines containing specific dominant nuclear genes which restore pollen fertility [3–5] resulted in a rapid production and cultivation of sunflower hybrids.

In a number of cases analysed, the CMS phenotype is suggested to originate from mutations in the mitochondrial genome of the male fertile progenitors as a result of intra- or intermolecular recombination events. The mitochondrial genome rearrangements have generated chimaeric mtDNA sequences which in some cases result in generation of novel mitochondrial genes or lead to a modification of existing genes [6]. These chimaeric genes are expressed as novel or modified polypeptides which, in an unknown fashion, are related to a failure in mitochondrial function during development of the pollen.

Our current knowledge about the molecular basis of CMS mainly comes from studies performed in maize and petunia. The chimaeric mitochondrial gene T-urf 13, composed primarily of sequences derived from the 26S rRNA, 18S+5S rRNA and coxII was investigated by Southern blot analysis. These genes have similar structures in fertile and all studied sterile sources. Using the atpA probe, 5 from the 6 investigated CMS genotypes showed identical hybridization patterns to the Petunia CMS line, which is used in all commercial sunflower hybrids. Only 1 cytoplasm derived from an open pollination of Helianthus annuus ssp. texanus, known as ANT, contained a unique mitochondrial DNA fragment, which is distinguishable from the fertile and sterile Petunia CMS genotypes and from all investigated CMS genotypes. Male fertility restoration and male sterility maintenance of the ANT line are different from the Petunia CMS system, which is a confirmation that a novel CMS genotype in sunflower has been identified.

Sunflower; Mitochondrial genome; Cytoplasmic male sterility; RFLP; atpA locus

1. INTRODUCTION

Cytoplasmic male sterility (CMS) is a maternally inherited trait in higher plants that results in the inability of the mature plant to produce functional pollen, but it does not affect female fertility [1]. In sunflower a CMS genotype was obtained from an interspecific cross between Helianthus petiolaris and H. annuus which was first described by Leclercq [2]. The subsequent identification of male fertile lines containing specific dominant nuclear genes which restore pollen fertility [3–5] resulted in a rapid production and cultivation of sunflower hybrids.

In a number of cases analysed, the CMS phenotype is suggested to originate from mutations in the mitochondrial genome of the male fertile progenitors as a result of intra- or intermolecular recombination events. The mitochondrial genome rearrangements have generated chimaeric mtDNA sequences which in some cases result in generation of novel mitochondrial genes or lead to a modification of existing genes [6]. These chimaeric genes are expressed as novel or modified polypeptides which, in an unknown fashion, are related to a failure in mitochondrial function during development of the pollen.

Our current knowledge about the molecular basis of CMS mainly comes from studies performed in maize and petunia. The chimaeric mitochondrial gene T-urf 13, composed primarily of sequences derived from the 26S rRNA, 18S+5S rRNA and atp6 gene, is unique for maize with Texas male sterile cytoplasm and codes for a 13-kDa polypeptide [7,8]. In reversion of CMS-T maize to fertility T-urf 13 is deleted or truncated through recombination [9–12]. Two dominant nuclear genes, Rf1 and Rf2, restore pollen fertility and reduce the abundance of the 13-kDa polypeptide [7]. In petunia the CMS phenotype was also shown to be associated with a specific DNA segment of the mitochondrial genome [13,14]. The DNA sequence and transcript pattern of the CMS-associated region, pef-S, were determined [15,16]. A 25-kDa protein associated with CMS in petunia was identified. One nuclear gene Rf1 is sufficient to confer fertility and reduce the abundance of this 25-kDa pef-S protein [17].

The use of only 1 sunflower CMS source on a large scale may lead to a reduction of the genetic variability of the breeding material and to genetic vulnerability to diseases. A convincing example of the latter is the maize Texas CMS which is susceptible to Southern corn leaf blight [18]. Obviously it is important to increase the cytoplasmic genetic diversity in crop plants by identi-
flying or creating new sources of male sterility and also to investigate the molecular, biochemical and physiologi-

cal basis of CMS. Little is known about the molecular determination of CMS in sunflower. In the restriction

game map of the mitochondrial DNA of sunflower an area of 17 kb, including the atpA gene, is different in the Pelti-

tarisis CMS line compared to its fertile analogues [19]. In this paper we present our study of 6 new sunflower

CMS genotypes. These genotypes were obtained from intra- or interspecific crosses of Helianthus species or

mutagenesis of sunflower cultivars, and have been characterized for further utilization in breeding pro-

grammes.

2. MATERIALS AND METHODS

2.1. Plant material

Plants were grown in the field and leaves, used for DNA isolation, were harvested before flowering. The origins of the different cyto-

plasmic male sterile genotypes are described in Table 1.

2.2. Isolation of mitochondrial DNA

50 g of leaf material was homogenized in 250 ml ice-cold buffer comprising 0.4 M mannitol, 50 mM Tris-HCl, 3 mM NAD+EDTA, 0.2% Poly-L-lys, 0.1% bovine serum albumin, 10 mM 2-mercapto-

ethanol, pH 8.0. The ruptured cells were filtered through cheesecloth and mirucloth. The debris mid crude chloroplast fraction were col-

lected at 3.000 x g for 15 min, followed by a centrifugalion at 18.000 x g for 20 min to sediment the mitochon-

dria. Mitochondrial pellets were resuspended in 1 ml TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and lysed with 100 μl 10% SDS and 100 μl 10% Sarkosyl during an incubation of 20 min at 65°C. For protein precipitation 100 μl ice-cold 5 M potassium acetate was added, followed by an incubation

for 20 min at 4°C. The precipitated protein complexes were removed by centrifugation at 12,000 x g for 10 min and the super-

nats were collected by filtration through cheesecloth for further purification by phenol-chloroform extractions and ethanol precipitations.

The nucleic acid pellets were dissolved in 0.4 ml sterile water and treated with RNAse 10 mg/ml for 30 min at 37°C. After 2 subsequent phenol-chloroform extractions and a final ethanol precipitation the mtDNA was resuspended in 100 μl TE.

Table 1

<table>
<thead>
<tr>
<th>Code</th>
<th>Fertility</th>
<th>Origins</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>HAa</td>
<td>F</td>
<td>H. annuus</td>
<td>Leroy et al. [27]</td>
</tr>
<tr>
<td>CMS50</td>
<td>S</td>
<td>H. petiolaris X H. annuus</td>
<td>Leroy et al. [27]</td>
</tr>
<tr>
<td>CMS71</td>
<td>S</td>
<td>H. argophyllus X H. annuus</td>
<td>Christov [28]</td>
</tr>
<tr>
<td>CMS81</td>
<td>S</td>
<td>γ irradiation of cultivar Hemus</td>
<td>(unpublished results)</td>
</tr>
<tr>
<td>CMS81</td>
<td>S</td>
<td>H. annuus ssp. reamenus, open pollination</td>
<td>Vranceanu et al. [26]</td>
</tr>
<tr>
<td>CMS61</td>
<td>S</td>
<td>sonication of cultivar Peredovik</td>
<td></td>
</tr>
<tr>
<td>CMS81</td>
<td>S</td>
<td>H. scabridus X H. annuus</td>
<td>Christov (unpublished results)</td>
</tr>
<tr>
<td>CMS61</td>
<td>S</td>
<td>H. annuus</td>
<td>Bohorova (unpublished results)</td>
</tr>
<tr>
<td>CMS61</td>
<td>S</td>
<td>H. hirsutus</td>
<td>Bohorova et al. [29]</td>
</tr>
</tbody>
</table>

F, fertile; S, sterile

2.3. DNA Analysis

About 2 μg mtDNA was digested with 20 U of restriction enzyme (Bethesda Research Laboratories), fractionated by electrophoresis on

a 1% agarose gel and blotted onto Hybond N+ by vacuum blotting (LKB). Hybridization with random priming labeled probes was

carried out in 10% dextran sulphate, 1 M NaCl, 1% SDS and 200 μg/ml denatured herring sperm DNA at 60°C. After washing down to 0.1 SSC at 60°C, blots were autoradiographed using Kodak X- 

Omat AR films.

The probes used in this analysis have been provided by Dr. Toru Terachi, Kyoto Sangyu University, Japan (personal communication). The following mitochondrial probes were used: atpA (1.5-kb HindIII-

EcoRI fragment, containing the coding region of subunit A of the ATPase gene of Petunia hybrida), cytb (1.9-kb EcoRI fragment, contain-

ing the coding region of the cytochrome c oxidase subunit II of Phaseolus vulgaris), 18S + 25S rDNA (a 3.2-kb BamHI Sall fragment which contains the 5' upstream region of the 18S and 25S rRNA genes and also the tRNA(Met) gene from Trifolium aestivum [20]); and 26S rRNA (a 5.2-kb BamHI-Sall fragment from Trifolium aestivum which contains part of the 5' upstream region of the 26S rRNA gene [21]).

3. RESULTS

Southern blot analyses were performed to investigate the mitochondrial genomes of several new sunflower CMS sources. Digested and electrophoretically separated mtDNA was blotted to nylon membranes and hybridized with different clones of mitochondrial genes. The results show that the atpA probe hybridized to DNA fragments of different sizes in fertile- (F) as compared to sterile- (S) and investigated new CMS

genotypes, when digested with the restriction endonu-

ceases BstEI and SalI (Fig. 1). The atpA probe hybridized to a 13.9-kb BstEI fragment and a 4.4-kb SalI fragment from the F line and to a 5.8-kb BstEI and 7.0-kb SalI fragment from the S line. Some weak hybridization can also be observed in addition to the main bands both in the F, S and CMS lines. This proba-

bly is due to short repeated sequences as has also previ-

ously been reported by Köhler et al. [22]. It was demonstrated that the hybridization pattern of five out of six investigated CMS sources is identical to the Peltiolaris sterile genotype, despite the fact that they were developed from different crosses between Helianthus species or by mutagenesis of sunflower cultivars and manifest different morphological and physiological characteris-


tics.

One of the investigated lines, CMS3, showed a different restriction fragment length polymorphism (RFLP) distinguishing both F- and S lines. The atpA probe hybridized to a 9.2-kb BstEI fragment and a 1.5-kb SalI fragment of CMS3 mtDNA indicated as NS (new sterile). Different hybridization patterns were also found when the atpA probe was hybridized to mtDNA from F-, S- and NS lines after digestion with the enzyme HindIII, BglII, PstI, BstEI/BglII, BstEI/Sall and HindIII/Sall. Fig. 2 presents the RFLPs between F-, S- and NS lines obtained after double digestions with BstEI/BglII-BstEI/Sall and using the atpA probe. The results from Southern blot analysis have been used to
create restriction maps of the \textit{atpA} area in mtDNAs from fertile HA$_{pet}$, sterile CMS$_{pet}$, and new sterile CMS$_{x}$ sunflower lines (Fig. 3). In this figure it is shown that to the left of the \textit{Sai} site in the \textit{atpA} gene the mtDNA organization of the F-, S- and NS lines is colinear. Differences in the mtDNA organization between F-, S- and NS lines are observed to the right of the \textit{Sai} site. These results emphasize the differences in genome organization between F-, S- and NS lines and exclude the probability of a point mutation in the mitochondrial genome of the NS sunflower line.

In order to further analyze the mtDNA organization in F-, S- and the described CMS lines, and to clarify whether rearrangements took place elsewhere in the mitochondrial genomes, we carried out Southern hybridization analyses with cloned mitochondrial genes from \textit{P. sativum} and \textit{T. aestivum} as heterologous probes. The genes used were \textit{coxII}, 26S rRNA, 18S + 5S rRNA. The results, shown in Fig. 4, indicate that \textit{coxII} hybridized to the mtDNA from F-, S- and (NS) lines double-digested with \textit{BstEII/BglII-BstEII/SaiI} and \textit{BstEII/SaiI} without any differences among the various lines. No differences were detected either in hybridization patterns using other enzymes like \textit{HindIII}, \textit{BgII}, \textit{PstI} and the other mitochondrial probes, including 26S rRNA, 18S+5S rRNA and \textit{coxII}. It is obvious that these regions have a similar structure in fertile and all sterile sources and probably are not involved in recombination events associated with CMS in sunflower.

4. DISCUSSION

At present all commercial sunflower hybrids contain the \textit{Petiolaris} CMS which was found by Leclercq [2]. In order to be able to introduce cytoplasmic diversity into sunflower we analyzed in this study the high molecular weight mtDNA of possible new types of sunflower CMS. Studies were conducted on 6 CMS genotypes and a near-isogenic male sterile and male fertile sunflower line in Southern blot analyses using 10 restriction endonucleases and 5 mitochondrial genes. The \textit{atpA} probe distinguished between the CMS S- and F-type in the hybridization experiments with all tested restriction enzymes. Out of the 6 investigated CMS genotypes 5 showed identical hybridization patterns indistinguishable from the well known \textit{Petiolaris} CMS. No specific restorer sunflower lines have been found for the different CMS genotypes. However, some \textit{Petiolaris} CMS maintainers partially restore male fertility of these sunflower CMS genotypes.

During preparation of this manuscript Crouzillat et al. [23] reported the genetic analysis and molecular basis of 15 sunflower CMS sources which have different origins than the genotypes included in our studies. In agreement with our results they found identical hybridization patterns indistinguishable from the well known \textit{Petiolaris} CMS. No specific restorer sunflower lines have been found for the different CMS genotypes. However, some \textit{Petiolaris} CMS maintainers partially restore male fertility of these sunflower CMS genotypes.
**Fig. 2.** Southern blot analysis of mtDNA isolated from a fertile HA*U (F), sterile (S) and CMS, (NS) line, obtained from an open pollination of *H. annuus* ssp. *leucaenas*. The mtDNA, double-digested with *BstE* II/Bgl I and *BstE* II/Sal I was hybridized with a random priming labeled *atp A* probe. The arrows indicate the CMS-specific polymorphisms between F-, S- and NS lines.

*atp A*, these results lead to the suggestion that probably, by being affected in the *atp A* area the different sunflower CMS genotypes are related.

The organization of the mitochondrial genomes of the *Patulares* CMS genotypes and all studied new CMS lines regarding the 4 other genes which have been examined (*cox II, 26S rRNA, 18S+5S rRNA*) is identical to the F-type. According to Crouzillat et al. [23] the genes

**Fig. 3.** Restriction maps of the surrounding regions of the *atp A* locus in fertile (F), sterile (S) and CMS, (NS) lines. Restriction sites shown: (T) *BstE* II; (B) *Bgl* I; (S) *Sal* I; (H) *Hind* III; and (P) *Pst* I. The maps have been created on the basis of Southern blot analysis and [19]. The arrow under the *atp A* gene indicates the direction of transcription according to [22].

26S rRNA. *18S+5S rRNA* show less variability than those coding for ATPases, but they could still find RFLP differences in 3 groups of CMS cytotypes. Using 3 enzymes and 12 probes per genotype they found 20% RFLPs (using the *26S rRNA* probe) and 33.4% RFLPs (using the *18S + 5S rRNA* probe) which differ from the F genotype [23]. In our study we tested 40 different enzyme/probe combinations (except *atp A*) for each CMS genotype and could not find any RFLP. No differences, not only for the *26S rRNA* and *18S + 5S rRNA* but also for the *cox II* gene, have been found, which is an indication that these loci are not involved...
in events associated with CMS. This does not exclude the possibilities of other RFLP differences elsewhere in the mitochondrial genomes, which could be hypothesized as additional deficiencies responsible for CMS. However, recent publications show that in the commonly used CMS sunflower genotype there is a correlation between CMS and co-transcription of a new open reading frame with the \textit{atpA} gene \cite{22,24}. Probably the translation product of this open reading frame is a 16-kDa polypeptide which is suggested to play a role in the CMS phenotype \cite{25}. This also points to the area of the \textit{atpA} locus to be involved in CMS in sunflower.

One of the investigated lines, CMS$_2$, obtained from an open pollination of \textit{H. annuus} ssp. \textit{texanus} and known as ANT \cite{26}, showed a different RFLP distinguishing this NS line from both F- and S genotypes and all new investigated sunflower CMS lines. This NS line was characterized by a complete anther and pollen atrophy and has proven to be stable under various environmental conditions. Classical restorers commonly used in the CMS system do not restore fertility of this NS line and up until now no sunflower lines have been found that restore the fertility of the NS line ([26], P. Petrov, unpublished results). These results agree with the molecular characterization and suggest the existence of a novel type of CMS in sunflower, originating from rearrangements in the vicinity of the \textit{atpA} gene. The \textit{atpA} probe in the investigations of Crouzillat et al. \cite{23} detected 8 cytotypes among 15 sunflower cytoplasms; apparently the \textit{atpA} gene is involved somehow in many sunflower CMSs. In order to understand the molecular basis of CMS in sunflower future experiments will concentrate on differences in organisation and expression of genes in the area of the \textit{atpA} locus between F-, S- and NS genotypes.

Acknowledgements: We like to thank Dr. Toru Terauchi, Kyoto Sangyin University, Japan, for providing the clones used as probes in the analysis of the mitochondrial genomes. We are grateful to Dr. P. Ivanov, Institute of Wheat and Sunflower, for providing M.S. with laboratory facilities for DNA isolation. We would like to thank Dr. Mark van Haaren for his comments on the manuscript.

REFERENCES

\begin{thebibliography}{99}
\bibitem{17} Nivison, H.T. and Hanson, M.R. (1989) Plant Cell 1, 1121-1130.
\end{thebibliography}